

**OSTEOPROMOTION OF MANDIBULAR
DISTRACTION OSTEOGENESIS USING STEM
CELLS FROM HUMAN DECIDUOUS TEETH AND
IN BIPHASIC CALCIUM PHOSPHATE
SCAFFOLD IN RABBIT MODEL**

AMERA KHALIL MOHAMMED .A

UNIVERSITI SAINS MALAYSIA

2013

**OSTEOPROMOTION OF MANDIBULAR DISTRACTION
OSTEOGENESIS USING STEM CELLS FROM HUMAN
DECIDUOUS TEETH AND IN BIPHASIC CALCIUM
PHOSPHATE SCAFFOLD IN RABBIT MODEL**

by

AMERA KHALIL MOHAMMED .A

**Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy**

May 2013

ACKNOWLEDGEMENTS

By the name of Allah The Most Merciful. Thank God for providing me patience and ability to perform this task to obtain a PhD degree in Oral and Maxillofacial Surgery.

Along the most important moments of our life, there are a lot of persons that pass and leave deep marks that we will never forget. This was definitely one of the moments in which I felt the strong presence of several persons to whom I cannot forget to acknowledge.

I could never have completed this thesis without the hard work and personal support of a great many people. None of the experiments could have been run without the technical assistance of many of my fellow scientists.

First I would like to thank both my universities, Al-Anbar, Iraq for the financial support during my study period, the President Professor Khalil Alduliami for his continuous support and encouragement, Professor Esam Alhadethi, Dr Hassan Alduliami and Professor Rafil Hamed, Dean of Al-Anbar College of Dentistry and University Sains Malaysia which give me a chance to study in such a renowned university in Malaysia.

My deepest gratitude goes to my supervisor and mentor Associate Professor Dr Noor Hayati Abdul Razak. Thanks for trusting me, leaving me the freedom to work alone at times. I am grateful for her leadership and friendship from which I have learned immensely.

Special thanks and appreciation to Professor. Abd. Rashid Ismail, the former dean and my co-supervisor for his continuous support.

My special thanks go to Prof Zainal Ahmad, my co-supervisor for giving me the opportunity of working in the fascinating world of biomaterials and for his efforts in guiding me through a steep learning curve of the techniques of material synthesis and characterization.

My great respect for him and all the staff of the School of Materials and Mineral Resources ,Universiti Sains Malaysia.

My great thanks and gratitude goes to my co supervisors Associate Professor Dr Sam'an Malik Masudi.

My great respect and thanks goes to Associate Professor Dr. Mutum Samarendra for his assistance in histopathological interpretation of my slides which was prepared efficiently by Mr Rosli Jusoh.

My special thanks goes to Professor Zulkifli Ahmad for his continuous support to all post graduate students. I never forget your kind help for assisting me in statistics, wish you continued success and may Allah bless you.

I would like to acknowledge all members of the craniofacial lab especially Puan Asiah Abu Baker and Siti Fadilah Abdullah for the efforts they have made in guiding me through the learning techniques in tissue culture. Eda Sharip taught me scanning in image analyzer. I would also like to thank Mr Marzuki Yusof , Mohd Yosof Soon

Abdullah, Chairul Sopian, Mohammad Ezany Yusoff, Khadijah Mohd Ali, Nora and Siti Zulaiha Khashbulla for their help.

My gratitude also goes to Mr Jasmani Ab Manaf for his patience for fabricating the distractors repeatedly with his wonderful art and skills.

I want to extend special thanks to all the staff of Animal Research and Service Centre (ARASC) of the Healthy Campus, University Sains Malaysia namely Dr. Rumaizi Shaari and my best assistance during operation Dr Noziah Ghani and Faizul Ismal Che Adam. Mr Maarof Salleh who had helped me when necessary. Mr Mohd Nor Adling Abdullah who helped me to take care of my rabbits, Mr Nur Muhamad Mohd Makhatar for providing me the animals and Mr Ahahrol Anuar Ab Latif , of which without their help I would be unable to do this work.

There are no words to express how much I am grateful to my family, my great love and thank to my brother Ismael Khalil and my sister Prof Samia Khalil for their persistent support over the years. They remain my teachers and the love and values they have given me will always guide me through my life.

Finally, I would like to dedicate this thesis to my country Iraq and my second home Malaysia. You are the reason for me to strive and work hard every day!

Dr. Amera Khalil Mohammed Alkaisi

TABLE OF CONTENT

Acknowledgment	ii
Table of Contents.....	v
List of Tables	viii
 List of Figures	 xiv
 List of Charts	 xvii
 List of Abbreviation.....	 xviii
 Abstrak.....	 xxii
 Abstract.....	 xxii
CHAPTER 1 INTRODUCTION	
1.1 Background.....	1
1.2 Tissue engineering.....	5
1.3 Gap statement.....	9
1.4 Aim of the study.....	11
1.5 Hypothesis.....	12
1.6 Justification of study	13
CHAPTER 2 LITERATURE REVIEW	
2.1 Distraction osteogenesis (DO).....	14

2.1.1	General history of distraction osteogenesis.....	14
2.1.2	History of DO in the maxillofacial complex.....	16
2.1.3	Ilizarov Principles	17
2.1.4	Biological Aspects.....	17
2.1.5	Histology.....	17
2.1.6	The molecular biology of distraction osteogenesis.....	18
2.1.7	Neoangiogenesis.....	27
2.1.8	Optimum parameters.....	29
2.1.9	Distraction devices.....	29
2.1.10	Classification of distraction devices used in oral and maxillofacial surgery...30	
2.1.11	Vector Selection.....	33
2.1.12	Phases of postsurgical treatment	34
2.1.13	Pathophysiology	38
2.1.14	Regenerate Monitoring.....	38
2.1.15	Current Usage: Midface.....	40
2.1.16	New clinical applications of DO	41
2.1.17	Complications.....	42
2.1.18	Advantage of DO.....	42
2.1.19	Disadvantages	44
2.1.20	Regenerate Enhancement in maxillofacial surgery during DO.....	45
2.2	Tissue engineering.....	49
2.2.1	Stem cells MSCs.....	50
2.2.1(a)	Embryonic stem cells	50
2.2.1 (b)	Somatic Stem Cells.....	51

2.2.1(c)	Embryonic stem cells versus somatic stem cell.....	51
2.2.1(d)	Origin and sources of MSC.....	52
2.2.1(e)	Stem cells property.....	53
2.2.1(f)	Therapeutic indication.....	57
2.2.1(g)	Stem cell niche.....	58
2.2.1(h)	Hematopoietic stem cells.....	59
2.2.1(i)	Dental pulp stem cells.....	60
2.2.1(j)	Stem cells from human exfoliated deciduous teeth (SHED).....	62
2.2.2	Scaffold	65
2.2.2 (a)	Advantages of synthetic bone scaffolds	66
2.2.2 (b)	Ideal scaffold	66
2.2.2 (c)	Specific requirements for biomimetic synthetic bone scaffold...66	
2.2.2(d)	Calcium phosphate ceramics (Ca-P).....	67
2.2.2 (e)	Biphasic calcium phosphate ceramics (BCP).....	68
2.3	<i>In vitro</i> cytotoxicity of MBCP	72
2.3.1	Biocompatibility.....	72
2.3.1(a)	Tests for <i>in vitro</i> cytotoxicity (Cell culture tests).....	75
2.3.1(b)	Genotoxicity.....	80
2.3.1(c)	Usage test.....	81
2.3.1(d)	In vivo biocompatibility.....	82
2.4	Animal model.....	83
CHAPTER 3 MATERIALS AND METHODS		
3.1	Synthesis and Characterization of MBCP.....	85
3.1.1	Materials.....	86
3.1.2	Preparation of BCP.....	86
3.1.3	Characterization.....	88
3.1.5	Fabrication of porous BCP.....	89

3.1.6	Characterization of porosity.....	90
3.1.7	Preparation of MBCP granules.....	90
3.2	Isolation, expansion and characterization of SHED.....	91
3.2.1	Materials.....	91
3.2.2	Preparation of primary culture.....	91
3.2.3	Cell culture and cellular morphology.....	93
3.2.4	Cell expansion.....	96
3.2.4 (a)	Cell passage.....	96
3.2.4 (b)	Cell counting and viability assessment using trypan blue	97
3.2.5	Characterization SHED by flow cytometry.....	98
3.3	<i>In vitro</i> cytotoxicity of MBCP.....	99
3.3.1	Target cells.....	99
3.3.2	Sterilization method.....	99
3.3.3	Preparation of extracts.....	100
3.3.4	Cell culture preparation.....	101
3.3.4(a)	Preparation of culture medium.....	101
3.3.4(b)	Cell culture technique.....	102
3.3.5	Preparation of cell strain.....	102
3.3.6	Cytotoxicity test (Extract dilution method).....	103
3.3.6 (a)	MTT assay.....	103
3.3.7	Cell viability/proliferation.....	107
3.3.8	Data collection and Statistical analysis.....	108
3.4	Methodology of the <i>in vivo</i> study	109
3.4.1	Study design.....	109
3.4.2	Animal care.....	109
3.4.3	Sample size calculation.....	110
3.4.4	Materials.....	111

3.4.5	Distractors.....	111
3.4.6	Housing and Feeding.....	112
3.4.7	Experimental Design.....	112
3.4.8	Anaesthesia.....	113
3.4.9	Prophylaxis	113
3.4.10	Analgesic.....	114
3.4.11	Preparation of the animal prior to surgery.....	114
3.4.12	Surgical procedure.....	114
3.4.13	Preparation of MBCP granules	117
3.4.14	Preparation of SHED.....	118
3.4.15	Cell seeding.....	118
3.4.16	Distraction osteogenesis protocol.....	119
3.4.17	Transplantation protocol.....	120
3.4.18	Cell survival in the construct.....	121
3.4.19	Post operative care	122
3.4.20	Latency period.....	122
3.4.21	Distraction period.....	123
3.4.22	Consolidation period.....	123
3.4.23	Follow up	123
3.4.24	Tissue harvesting	124
3.4.25	Blocks preparation	124
3.4.26	Clinical Evaluation (Macroscopic Examination).....	125
3.4.27	Radiographic Examination (Soft X-Ray).....	126
3.4.28	Histological examination.....	127
3.4.29	Histomorphometric examination.....	127

CHAPTER 4 RESULTS

4.1	Synthesis and characterization of microporous biphasic calcium phosphate	132
-----	--	-----

4.1.1	Synthesis of MBCP	132
4.1.2	Characterization.....	133
4.1.2(a)	X ray diffraction (XRD).....	133
4.1.2(b)	Scanning electron microscope (SEM) analysis	134
4.1.2(c)	Particle size analysis.....	135
4.1.3	Porosity of MBCP results.....	136
4.1.3 (a)	Micro porosity.....	136
4.1.3 (b)	Macroporosity.....	136
4.1.4	Granules preparation result.....	137
4.2	<i>In vitro</i> isolation expansion and characterization of SHED.....	137
4.2.1	Cellular morphology in passage 0	138
4.2.2	Cellular morphology in passage 1.....	139
4.2.3	Cellular morphology in passage 7.....	140
4.2.4	Cell yield in the primary (passage 0) cell cultures.....	140
4.2.5	Cell yield in the primary (passage 7) cell cultures.....	141
4.2.6	Flow cytometry identification of SHED.....	141
4.3	<i>In vitro</i> cytotoxicity of MBCP.....	143
4.3.1	Test on extract results.....	143
4.3.2	Statistical analysis.....	145
4.4	<i>In vivo</i> study (DO osteopromotion).....	149
4.4.1	Cell survival in the treatment construct.....	149
4.4.2	Clinical evaluation.....	150
4.4.4	Radiological Observation	154
4.4.4	Histological findings.....	161
4.4.5	Histomorphometric results	173

CHAPTER 5 DISCUSSION

5.1 BCP synthesis	189
5.1.1 HA / β -TCP ratio.....	190
5.1.2 Microporosity.....	191
5.1.3 Macroporosity.....	192
5.1.4 The total porosity percentage.....	195
5.1.5 Granules.....	196
5.2 Isolation, expansion and characterization of SHED.....	197
5.2.1 Isolation and expansion.....	197
5.2.2 Characterization of SHED using Flow cytometry.....	200
5.2.3 Cell morphology and growth kinetics of the SHED.....	201
5.2.4 Determination of cell yield.....	202
5.3 Biocompatibility of MBCP.....	203
5.3.1 <i>In vitro</i> cytotoxicity study.....	204
5.3.2 SHED cell primary culture.....	207
5.3.3 Test on extract.....	209
5.4 <i>In vivo</i> osteopromotion.....	212
5.4.1 Choice of DO.....	214
5.4.2 Tissue engineering.....	215
5.4.3 Timing the implantation.....	231
5.4.4 Cell survival.....	234
5.4.5 Immunosuppression.....	236
5.4.6 Clinical observation.....	238
5.4.7 Radiography of the mandible	253
5.4.8 Histology of the distracted regenerate.....	241

5.4.9 The histomorphometry of the rabbit DO model.....	250
5.4.10 The importance of the vascularity and paracrine growth factors to cell survival.....	256
5.4.11 Stem cells from human deciduous teeth performance in the rabbit study.....	257
5.4.12 MBCP in rabbit DO study.....	258
5.4.15 Time possibly gained by this study.....	258
CHAPTER 6 Conclusion, Limitation of the study and Future direction	
6.1 Conclusion	259
6.2 Limitation of the study.....	261
6.3 Future direction.....	263
References	267
APPENDICES.....	323
Appendix I The Ethical Approval	
Appendix II Academic Activities	
List of Publications & Presentations	

LIST OF TABLES

CHAPTER 3 MATERIALS AND METHODS

Table 3.1 Composition of media used in the <i>in vitro</i> and <i>in vivo</i> studies.....	95
Table 3.2 Bone formation grading scores for radiographic analysis	

	(Yasko <i>et al.</i> , 1992 and Kirker-Head <i>et al.</i> , 1995).....	127
Table 3.3	Histological scoring system based on different factors in healing Process (Heiple <i>et al.</i> , 1964 and modified by Linde and Hedne, 1995).....	131
CHAPTER 4 RESULTS		
Table 4.1	Particle size analysis result of BCP synthesis by wet precipitation with Heating.....	135
Table 4.2	Mean optical absorbance (OD) and relative growth Rate (RGR) of SHED (Experiment 1 and 2).....	142
Table 4.3	Comparing OD between all MBCP concentrations and control (1 st experiment).....	146
Table 4.4	Comparing OD variables between each BCP concentrations and control (1 st experiment).....	147
Table 4.5	Comparing OD between all BCP concentrations and control (2 nd experiments).....	148
Table 4.6	Comparing OD between all BCP concentrations with 3 day and 7 days extraction periods.....	149
Table 4.7	Showing the relation between pre & post operative weight.....	154
Table 4.8	Bone formation grading scores results for radiographi analysis (Yasko <i>et al.</i> , 1992 and Kirker-Head <i>et al.</i> , 1995).....	161
Table 4.9	Percentage of newly formed bone in group A, B and C	183
Table 4.10	Stage of bone union scores for group A,B and C.....	185
Table 4.11	Grade of bone maturity scores for group A , B and C.....	187

LIST OF FIGURES

CHAPTER 3 MATERIALS AND METHODS

Figure 3.1	Flow chart represent preparation of BCP by wet precipitation technique.....	87
Figure 3.2	Human pulp tissue harvest: Primary cell culture SHED.....	94
Figure 3.3	SHD cells counting A-Haemocytometer, B Cells under microscope... ..	98
Figure 3.4	MBCP samples.....	100
Figure 3.5	MBCP incubated with shaking.....	100
Figure 3.6	Extract supernatant filtered through a membrane.....	101
Figure 3.7	SHD cells cultured in T-75 tissue culture flask (experiment- 2).....	102

Figure 3.8	Three sets of 96-well plates with 3 samples of MBCP extract (1 st experiment).....	105
Figure 3.9	Two sets of 96-well plates with 2 samples of MBCP extract (experiment 2).....	105
Figure 3.10	MTT solution.....	106
Figure 3.11	Directly after the addition of MTT.....	107
Figure 3.12	Addition of DMSO and insertion in to spectrophotometer.....	108
Figure 3.13	Internal distractor.....	112
Figure 3.14	Mental nerve and osteotomy site	116
Figure 3.15	Placement of the distraction device.....	116
Figure 3.16	A- Sterilized MBCP granules B- MBCP in MEM alpha media C- MBCP+SHD MEM alpha.....	117
Figure 3.17	SHD 6×10^6	118
Figure 3.18	Protocol methodology.....	120
Figure 3.19	Remnant of construct in 10 cm ² tissue culture plate.....	121
Figure 3.20	The ROIs used for the histomorphometry analysis A- total ROI 5x magnification B- area of new bone.....	129
CHAPTER 4	RESULTS	
Figure 4.1	MBCP pellets.....	133
Figure 4.2	MBCP granules.....	133
Figure 4.3	XRD of calcined BCP showing the 2 phase HA (blue) and β - TCP (red).....	134
Figure 4.4	SEM of BCP (5.00kx).....	135
Figure 4.5	SEM picture of MBCP showing the microporosity.....	136
Figure 4.6	SEM pictures of MBCP showing the macroporosity.....	137

Figure 4.7	The cell morphology of primary culture (passage zero) viewed under light microscope (magnification x10),A-SHD colonies on day 5 and B-SHD morphology day 10.....	138
Figure 4. 8	The cells morphology (Passage one) viewed under light microscope (magnification x10).....	139
Figure 4.9	The cells morphology on (Passage seven) viewed under light microscope (magnification x10.....	140
Figure 4.10	Flow cytometry analysis for SHD showed positive expression against : A- CD 105 (42%) and B- CD166 (95%).....	142
Figure 4.11	Cell survival in the treatment construct, SHD/MBCP in the cell culture at 1,2,7 and 14 days after surgery.....	150
Figure 4.12	Inflammatory response A&B: Second and 3 rd day of operation	152
Figure 4.13	Post distraction deviation	153
Figure 4.14	Result of lengthening 6 mm.....	153
Figure 4.15	Specimens showing distracted area: A, B and C group A; D,E and F group B, and G, H and I group C, 2 ,4 and 6 weeks post operative.....	15
Figure 4.16	Radiograph of distracted area; A control, B SHD and C SHD /MBCP groups 2 weeks postoperative.....	156
Figure 4.17	Radiograph of distracted area; A control, B SHD and C SHED/MBCP groups 4 weeks postoperative.....	158
Figure 4.18	Radiograph of distracted area; A control, B SHD and SHD/MBCP groups 6 weeks postoperative.....	160
Figure 4.19	Histological observation, DO 2weeks group A.....	163
Figure 4.20	Histological observation, DO 2weeks group B.....	164
Figure 4.21	Histological observation, DO 2weeks group C.....	165

Figure 4.22	Histological observation of the DO 4 weeks group A.....	166
Figure 4.23	Histological observation of the DO 4 weeks group B.....	167
Figure 4.24	Histological observation of the DO 4 weeks group C.....	168
Figure 4.25	Histological observation of the DO 6 weeks group A.....	169
Figure 4.26	Histological observation of the DO in 4 weeks group B.....	170
Figure 4.27	Histological observation of the DO in 4 weeks group.....	171
Figure 4.28	Haematoxillin &Eosin staining of the DO area of all treatment groups 2 to 6 weeks; 5 x magnification, all images present the central area of DO.....	172

LIST OF CHARTS

Chart 4.1	The semi quantitative analysis of the woven bone produced in DO regenerate for the treatment groups which employed SHD alone or in combination with MBCP and empty DO. The data expresses the woven bone (trabeculi marrow was excluded) as a percentage of the total bone tissue seen in DO regenerate.....	174
Chart 4.2	Percentage of bone within DO regenerate, across all treatment groups at 2 weeks (a) control (b) SHD and (c) SHD/MBCP groups at 2 weeks (a) control (b) SHD and (c) SHD/MBCP.....	175
Chart 4.3	Percentage of bone within DO regenerate, across all treatment groups at 4 weeks (a) control (b) SHD and (c) SHD/MBCP.....	176
Chart 4.4	Percentage of bone within DO regenerate, across all treatment groups at 6 weeks (a) control (b) SHD and (c) SHD/MBCP.....	177

LIST OF ABBREVIATIONS

BCP	Biphasic calcium phosphate
MBCP	Macroporous biphasic calcium phosphate
BMPs	Bone morphogenetic proteins
BMMSCs	Bone marrow mesenchymal stem cell
CT	Computed tomography
ECM	Extra cellular matrix
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor
IGF-1	Insulin-like growth factor-1
MSCs	Mesenchymal stem cells
MTT	Dimethylthiazol diphen
OC	Osteocalcin
ON	Osteonectin

**KAJIAN PENGALAKKAN PERTUMBUHAN TULANG MENGGUNAKAN
SEL STEM DARIPADA GIGI DESIDUS DAN SEL STEM BERSAMA
KERANGKA BIPHASIC KALSIUM PHOSFAT DWIFASA DI DALAM
OSTEOGENESIS REGANGAN MANDIBEL MENGGUNAKAN MODEL
ARNAB**

ABSTRAK

Osteogenesis Regangan (OR) yang digambarkan sebagai kejuruteraan tisu tulang dalaman telah menjadi semakin popular di tahun-tahun kebelakangan ini dan teknik penggunaan telah mengalih perhatian kepada rangka kraniofasial yang telah mengembangkan bilangan rawatan alternatif untuk pesakit yang tidak normal dan kekurangan maksilofasial. Ia telah digunakan pertama kali dalam pembedahan ortopedik

untuk pembetulan kerencatan pertumbuhan anggota badan, dan seterusnya telah digunakan dalam rawatan microsomia kraniosfasial dan kecacatan tulang. Dalam OR, pembentukan tulang yang baru adalah didorong oleh pemisahan secara beransur-ansur segmen bertulang selepas osteotomi atau kortikotomi semasa proses biasa penyembuhan patah yang diganggu oleh penggunaan daya tarikan secara beransur-ansur terhadap kalus lembut. Walaupun OR mempunyai beberapa kelebihan yang nyata berbanding kaedah pembedahan tradisional terutamanya kedua-dua tisu yang lembut dan keras yang akan memanjang pada masa yang sama, namun kelemahan utama adalah rawatan yang lama yang kadangkala tidak dapat diterima oleh pesakit dan boleh dikaitkan dengan komplikasi bertulang dan tisu lembut dan kemungkinan

penyatuan berserabut atau ketidaksatuan kekal sebagai batasan utama yang menghalang amalan klinikal yang selanjutnya. Banyak kajian telah memberi tumpuan kepada promosi pembentukan tulang baru untuk memendekkan siri gangguan osteogenesis. Cara fizikal telah dimasukkan ke medan elektromagnetik berdenyut, keamatan ultrabunyi rendah dan rangsangan elektrik. Kaedah intervensi seperti pemindahan daripada sel-sel seperti osteoblast atau sumsum tulang ke bahagian gangguan, suntikan faktor pertumbuhan atau plasma platelet yang padat dan terapi gen juga telah digunakan untuk mempercepatkan kematangan tulang yang dihasilkan. Beberapa kaedah bukan intervensi, seperti penggunaan asid kalsitonin, alendronate dan zoledronik juga telah menampakkan kejayaan awal. Kejuruteraan tisu (KT) adalah satu bidang baru yang sangat menjanjikan rekonstruktif yang menarik dalam kemajuan terkini dalam bidang perubatan dan pembedahan. Tiga elemen utama KT adalah sel

stem, randuk dan faktor pertumbuhan. Sel stem dari gigi daun luruh exfoliatif (SHED) telah terbukti mampu dibezakan kepada osteoblas dan kondrosit-kondrosit dalam pembentukan tulang vitro dan in vivo dan menjadi sumber sel utama bagi kejuruteraan tisu tulang. Ia telah digunakan dalam pembinaan semula kecacatan saiz kritikal. Penggunaan SHED semasa gangguan osteogenesis untuk menggalakkan pertumbuhan tulang baru belum dilaporkan. Microporus dwifasa kalsium fosfat seramik (MDKF) merupakan biomaterial yang digunakan sebagai pengisi tulang dan sebagai tangga-tangga dalam kejuruteraan tisu tulang. Tujuan utama kajian ini adalah untuk menguji sama ada penambahan SHED dan komposit yang terdiri daripada pilihan dalam granul MDKF dalam osteotomy sebagai kejuruteraan tisu membina potensi peningkatan osteogenik dalam menjana semula OR. MBCP disintesis dengan ciri-ciri yang diinginkan, dicirikan dan diuji dalam cytotoxicity vitro. SHD adalah terencil, berkembang dan dicirikan. Lapan belas arnab putih New Zealand telah dibahagikan kepada 3 kumpulan. Kumpulan A merupakan kumpulan kawalan (tiada intervensi dalam potongan osteotomy), kumpulan B, 6 juta sel-sel (SHD) telah dipindahkan dan kumpulan C, (SHD / BCP membina) telah dipindahkan ke dalam potongan osteotomy. Janaan semula itu dinilai dalam 3 tempoh; 2,4 dan 6 minggu secara postoperatif menggunakan X-ray konvensional, bahagian histologi dan ukuran histomorfometrik. Jumlah tulang yang baru ditentukan kuantitinya menggunakan penganalisis imej Hasilnya menunjukkan bahawa kedua-dua SHD dan SHD / MBCP membina meningkatkan pembentukan tulang. Peratusan tulang yang baru tumbuh pada 2 minggu kawalan, SHD dan SHD /MBCP adalah 18,41, 41,53 dan 57,28, pada minggu 4 31,68, 59,78 DAN 66,49 DAN DALAM MINGGU 6 52.34,60.24 dan 72,98%. Kesimpulannya, kajian ini boleh memberikan maklumat tambahan dan bukti daripada pelbagai mekanisme tindakan SHD

dalam pembentukan tulang yang mungkin berguna dalam memilih tulang yang berkesan menggalakkan binaan untuk pembentukan tulang. Seperti yang telah ditunjukkan, kedua-dua SHD dan SHD / MBCP mempunyai aktiviti osteopromosi dan potensi binaan pembentukan tulang. Kajian lanjut tentang kesan SHD dan SHD / MBCP kekal penting kerana ia akan merentas laluan dalam membangunkan binaan osteopromotik untuk regangan osteopromosi manusia. MDKF disintesiskan dengan ciri-ciri yang diinginkan, diberi ciri-ciri dan diuji dalam cytotoxicity vitro. SHD adalah terpenencil, berkembang dan diberi ciri-ciri. Lapan belas Neozeanald putih arnab telah dibahagikan kepada tiga kumpulan. Kumpulan A kawalan (campur tangan tidak digunakan dalam pembedahan osteotomy, kumpulan B, 6 juta sel-sel (SHD) telah dipindahkan dan kumpulan C, SHD / MBCP membina telah dipindahkan ke dalam potongan osteotomy. Penilaian semula itu dinilai dalam 3 tempoh 2,4 dan 6 minggu secara post operatif menggunakan Xray konvensional, bahagian histologi dan ukuran histomorfometrik. Jumlah tulang yang baru dibilang menggunakan penganalisis imej. Hasilnya menunjukkan bahawa kedua-dua SHD dan SHD/ MBCP membantu meningkatkan pembentukan tulang. Peratusan tulang yang baru dibentuk pada 2 minggu kawalan, SHD I dan SHD / MBCP 18,41, 35.97 dan 57,28, pada minggu 4 31,68, 59,78 dan 66,49 dan dalam minggu 6 52.34,60.24 dan 72,98%. Kesimpulannya, kajian kami boleh memberikan maklumat tambahan dan bukti daripada pelbagai mekanisme tindakan SHD dalam pembentukan tulang yang mungkin berguna dalam memilih tulang yang berkesan menggalakkan pembinaan untuk pembentukan tulang. Seperti yang telah ditunjukkan kedua-dua SHD dan SHD / MBCP mempunyai aktiviti osteopromosi dan tulang yang berpotensi membentuk pembinaan. Kajian lanjut tentang kesan SHD kedua-dua SHD/ BCP kekal penting kerana

kita boleh membangunkannya sebagai pembinaan osteopromotik untuk gangguan osteogenesis manusia.

**OSTEOPROMOTION OF MANDIBULAR DISTRACTION OSTEOGENESIS
USING STEM CELLS FROM HUMAN DECIDUOUS TEETH AND IN
BIPHASIC CALCIUM PHOSPHATE SCAFFOLD IN RABBIT MODEL**

ABSTRACT

Distraction osteogenesis (DO) is described as endogenous bone tissue engineering has become increasingly popular in recent years and the application of distraction technique to the craniofacial skeleton has expanded the number of treatment alternatives for patients with maxillofacial abnormalities and deficiencies. It is applied first in orthopedic surgery for correction of limb length discrepancies, and subsequently has been utilized in the treatment of craniofacial microsomia and bony defect. In DO, new bone formation is induced by gradual separation of bony segments after an osteotomy or corticotomy during which the normal process of fracture healing is interrupted by the application of gradual traction to the soft callus. DO has some distinct advantages over

traditional surgical methods which involve the elongation of both hard and soft tissue at the same time. The major disadvantage is the long treatment time which is some time not well tolerated by patients and the chance of associated bony and soft-tissue complications with possible fibrous union or nonunion remains as major limitations impeding its further clinical application. Many studies have focused on the promotion of new bone formation to shorten the course of DO. Physical means have included pulsed electromagnetic fields, low- intensity ultrasound and electrical stimulation, interventional methods such as transplantation of osteoblast-like cells or bone marrow to the distraction site. Bone formation *in vivo* has become the main cell source for bone tissue engineering, it has been used in critical size defect reconstruction. The application of stem cells from exfoliative deciduous teeth (SHED) during distraction, injection of growth factors or platelet rich plasma and gene therapy have also been applied to accelerate the maturation of the regenerated bone. Some non interventional methods, such as administration of calcitonin, alendronate and zoledronic acid have also shown promising results. Tissue engineering (TE) is a new highly promising field of reconstruction that is drawing attention in recent advances in medicine and surgery. The main 3 element of TE are stem cells, scaffold and growth factor. SHED has been proven to be capable of differentiating into osteoblasts and chondrocytes *in vitro*. The usage of stem cells from human deciduous teeth (SHD) to promote new bone formation in DO has yet not been reported. Macroporous biphasic calcium phosphate ceramic (MBCP) is biomaterial used as bone filler and as a scaffold in bone tissue engineering. The main aim of this study was to test whether the addition of SHD and a composite consisting of SHD seeded in MBCP granules in osteotomy as a tissue engineering construct increases osteogenic potential in DO. MBCP was synthesized with desirable properties, Calcium /

Phosphate ratio, micro and macro porosities and particle size. The material was characterized using x ray diffraction, scanning electron microscope and particle size analyzer. *In vitro* cytotoxicity was performed to test the biocompatibility of the synthesized macroporous biphasic calcium phosphate. Stem cells were isolated from human pulp of deciduous teeth, expanded *in vitro* and characterized using 2 antibodies CD 166 and 105. The *in vivo* study was performed using rabbit's model, Eighteen New Zealand white rabbits were divided into 3 groups. Group A underwent DO without addition of materials as control, group B had 6 million cells (SHD) transplanted in osteotomy gap and group C had SHD/BCP construct consisted of 6 million cells in 50 mg MBCP transplantation. DO protocol was 4 days latency period, 6 days distraction period 1mm/day and assessed in 3 consolidation periods 3, 18 and 32 days. The regenerate was evaluated in 3 intervals of 2, 4 and 6 weeks postoperative periods, clinically, radiographically using conventional X ray, significance of callus formation, formation of bone cortex and bone marrow cavity were evaluated to compare between groups. Histological sections for new bone formation, blood vessels, cartilage and fibrous tissue were assessed. Quantitative histomorphometric measurements were carried out using Zeiss image analysis system to quantify the amount of bone formed, cartilage and fibrous tissue ruminants. The samples were also analyzed histomorphometrically for the grade of osseous regeneration using an established numerical scoring system for the assessment of bone healing. Serial sections were scored for 2 independent bone-forming indices stage of bone union and grade of bone maturity. The result demonstrated that MBCP synthesized with Ca/P ratio of 1.52 confirmed by XRD, micro and macro porosity of 200-400 μm was confirmed by SEM. *In vitro* cytotoxicity showed that MBCP is free of toxicity, Mann-Whitney test detected that the

optical absorbance (OD) median of SHD for all MBCP concentrations was statically higher than control using 7 days extraction, P value < 0.001 for each. SHD were successfully isolated by enzyme digestion method. Early cell cultures revealed typical fibroblast-like spindle shaped cells arranged in colonies. Results of flow cytometry showed expression of CD 105 and 166, >42 and >95% respectively. The *in vivo* study showed that both SHD and SHD/MBCP construct enhance bone formation in all time points 2, 4 and 6 weeks postoperatively. Radiographic examination revealed presence of radiological evidence of DO healing in all treatment groups with more bone formation in the transplanted one. Histologically, intramembrance ossification was evidence, the highest bony formation was seen in SHD/MBCP group followed by SHD group and the least amount was in control group. Histomorphometric measurement detected that the percentage of newly formed bone in 2 weeks control, SHD and SHD/BCP were 18.41, 35.97 and 57.28% respectively, in week 4 were 31.68, 59.78 and 66.49% and in week 6 were 52.34, 60.24 and 72.98% respectively. Non parametric ANOVA (Kruskal Whalis Test) showed significant difference between groups P value = 0.003. Bone-forming indices such as stage of bone union and grade of bone maturity were highest in SHD/BCP group and lowest in control group, Kruskal Whalis Test showed significant difference between groups P value = 0.001 and 0.002 respectively. In conclusion, this study may provide additional information and evidence of the various mechanisms of action of SHD in bone formation which may be useful in selecting effective bone promoting construct for bone formation. As we have shown both SHD and SHD/BCP have osteopromoting activities and potential bone forming construct. The osteopromoting effect is better with combination of SHD and BCP scaffold. Further studies on the effect of both SHD and SHD/BCP remain important as it may lead to

developing an osteopromotic construct for human distraction osteogenesis and to know whether SHD differentiate directly to form osteoblast or indirectly promote the bone formation in DO.

CHAPTER ONE

INTRODUCTION

1.1 Background

Reconstruction of bone deficiency such as craniofacial anomalies and complex trauma can pose serious problems in clinical surgery, including orthopaedic, plastic/reconstructive and craniofacial surgery. Successful bone repair depends on a sequential interaction of a tissue matrix scaffold with pluripotent cells and growth factors within the local environment. Only autogenous cancellous bone graft possesses all the important qualities of osteoinduction, osteoconduction, osteointegration and osteogenesis. Even though the healing of the autologous cancellous bone graft can be unpredictable, it is still considered the best material available to repair significant bone defects (Bauer and Muschler, 2000). Bone autograft is a graft of own cancellous bone tissue. However, the harvest of an autograft has several limitations, it requires a separate surgical approach with all its potential complications including prolonged surgery and anaesthetic time, donor site morbidity and pain, inadequate amount and quality of autograft (Ahlmann *et al.*, 2002; Boone, 2003).